Mater. Res. Soc. Symp. Proc. Vol. 845 © 2005 Materials Research Society AA5.4

# Biomedical Applications of Gold Nanoparticles Functionalized Using Hetero-Bifunctional Poly(ethylene glycol) Spacer

**Wei Fu[1], Dinesh Shenoy[2], Jane Li[3], Curtis Crasto[3], Graham Jones[3], Charles Dimarzio[4], Srinivas Sridhar[1], and Mansoor Amiji[2]**

[1]Department of Physics, [2]Department of Pharmaceutical Sciences, [3]Department of Chemistry and Chemical Biology, [4]Department of Electrical and Computer Engineering and the Keck Microscope Facility, Northeastern University, Boston, MA 02115

## Abstract

To increase the targeting potential, circulation time, and the flexibility of surface-attached biomedically-relevant ligands on gold nanoparticles, hetero-bifunctional poly(ethylene glycol) (PEG, MW 1,500) was synthesized having a thiol group on one terminus and a reactive functional group on the other. Coumarin, a model fluorescent dye, was conjugated to the PEG spacer and gold nanoparticles were modified with coumarin-PEG-thiol. Surface attachment of coumarin through the PEG spacer decreases the fluorescence quenching effect of gold nanoparticles. The results of cellular cytotoxicity and fluorescence confocal analyses showed that the PEG spacer modified nanoparticles were essentially non-toxic and could be efficiently internalized in the cells within one hour of incubation.

## Introduction

Nanotechnology offers unique approaches to probe and control a variety of biological and medical processes that occur at nanometer length scales, and is expected to have a revolutionary impact on biology [1] and medicine [2]. Among the approaches for exploiting nanotechnology in medicine, nanoparticles offer some unique advantages as sensing, image enhancement, and delivery agents [3,4]. Several varieties of nanoparticles with biomedical relevance are available including, polymeric nanoparticles, metal nanoparticles, liposomes, micelles, quantum dots, dendrimers, and nanoassemblies. To further the application of nanoparticles in disease diagnosis and therapy, it is important that the systems are biocompatible and capable of being functionalized for recognition of specific target sites in the body after systemic administration.

Nanoparticles based on gold chemistry have attracted significant research and practical attention recently. They are versatile agents with a variety of biomedical applications including use in highly sensitive diagnostic assays [5], thermal ablation and radiotherapy enhancement [6,7], as well as drug and gene delivery [8]. For instance, antibody-modified gold nanoparticles when used for detection of prostate specific antigen, had an almost a million-fold higher sensitivity that conventional ELISA-based assay [9]. Near-infrared radiation absorbing gold-silica nanoshells have been prepared and evaluated for thermal ablation of tumors after systemic administration [6].

For biomedical applications, surface functionalization of gold nanoparticles is essential in order to target them to specific disease areas and allow them to selectively interact with cells or biomolecules. Surface conjugation of antibodies and other targeting moieties is usually achieved by adsorption of the ligand to the gold surface. Surface adsorption, however, can denature the

proteins or, in some cases, limit the interactions of the ligand with the target on the cell surface due to steric hinderance. Additionally, for systemic applications, long-circulating nanoparticles are desired for passive targeting to tumors and inflammatory sites. Poly(ethylene glycol) (PEG)-modification of nanoparticles affords long circulating property by evading macrophage-mediated uptake and removal from the systemic circulation [10]. Surface modification of gold nanoparticles through the PEG spacer would, therefore, allow the modified nanoparticles to remain in the systemic circulation for the prolonged period and provide flexibility to the attached ligand for efficient interaction with its target.

In the present study, we describe the synthesis of hetero-bifunctional PEG with a thiol group on one terminus and a reactive functional group on the other for conjugation to a biologically-relevant targeting moiety. Using thiol-PEG-coumarin, a model fluorescent dye, we describe the "proof of concept" for surface functionalization of gold nanoparticle, cellular cytotoxicity evaluations, and fluorescence confocal analysis of cell uptake and nanoparticle distribution.

## Experimental Methods

*Synthesis and Characterization of Coumarin-PEG-Thiol:* The hetero-bifunctional polyethylene glycol (PEG) compound containing end-groups thiol and alcohol derivatized as coumarin carbamate was efficiently prepared. The key step for the PEG synthesis involves the desymmetrization of the PEG-diol compound. This was achieved by monotosylation of the PEG-diol (MW 1,500 daltons) purchased from Aldrich (Milwaukee, WI) using the method of Bouzide and Sauve [11]. Thus, a solution of the PEG-diol in methylene chloride containing freshly prepared silver(I) oxide (1.5 equiv.), catalytic potassium iodide (0.2 equiv) and recrystallized *p*-toluenesulfonyl chloride at room temperature gave the monotosylate PEG derivative with 100% selectivity and with high yield (95%). This reaction could be done on a large-scale (20-50 g), yielding multigram quantities of the PEG product. None of the bistosylate PEG derivative was observed by alumina thin-layer chromatography. This later compound could be prepared alternatively and in 96% yield by treatment of the PEG-diol with 10 equivalents of tosyl chloride in pyridine heated at 60°C.

Refluxing a solution of the monotosylate PEG compound and potassium thiosulfate in dry methanol gave the PEG-thioacetate in 90% yield after chromatography and a single crystallization. Nucleophillic addition of the hydroxy-PEG-thioacetate to the coumarin-isocyanate reagent gave the coumarin carbamate-PEG-thioacetate in 85% yield. Finally, deprotection of the thioacetate to give the coumarin carbamate-PEG-thiol was achieved by base hydrolysis at room temperature using sodium methoxide in degassed methanol. Acidification of the reaction with Dowex DR-2030 cation exchange resin followed by filtration gave the thiol product in 76% yield. After purification, the coumarin-PEG-thiol was verified by mass spectroscopy and $^1$H-NMR.

*Preparation and Characterization of Gold Nanoparticles:* Gold nanoparticles were synthesized by reduction of gold chloride ($HAuCl_4$) with freshly prepared sodium citrate and allowed to boil under reflux conditions. The pale yellow-colored solution turned to deep red as the gold nanoparticles were formed and stabilized by adsorbed chloride ions. Particle size analysis was performed with a Coulter® N-4 sub-micron particle size analyzer (Coulter Corporation, Miami,

FL). The nanoparticles suspension was air-dried on the specimen grid and observed with a JEOL JEM-1010 transmission electron microscope (TEM). The accelerating voltage was set to be 60 KV and the specimen was observed at the original magnification of 250,000X. Based on our experience this method reproducibly produces spherical monodispersed nanoparticles.

*Surface Functionalization of Gold Nanoparticles with Coumarin-PEG-Thiol:* Coumarin-PEG-thiol, prepared as described above, was added to the aqueous dispersion of gold nanoparticle and the reaction for conjugation of the fluorescence dye to the gold surface through the thiol functionality was carried out at room temperature as shown in Figure 1. Fluorescent conjugated nanoparticles were separated from free coumarin-PEG-thiol by centrifugation and washing steps. Attachment of coumarin on gold nanoparticles was confirmed by fluorescence spectroscopy of the conjugated nanoparticles.

*Cytotoxicity Studies:* The *in vitro* cytotoxicity of the coumarin-PEG-thiol functionalized gold nanoparticles was measured using a commercially available CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit, purchased from Promega (Madison, WI). According to the manufacturer's instructions, the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, (MTS) is bioreduced by viable cells into a formazan product. Approximately 10,000 MDA-MB-231 human breast carcinoma xenograft cells, suspended in Dulbecco's minimum essential medium (DMEM) modified with 10% (v/v) fetal bovine serum and other essential nutrients, were seeded per well into 96-well plates and allowed to adhere for 24 hours at 37°C and 5% $CO_2$ atmosphere. The medium was replaced with graded concentrations of polyethyleneimine (MW ~ 10,000) as a positive control; unfunctionalized gold nanoparticles and nanoparticles functionalized with methoxy-PEG-thiol (OMe-PEG-SH) as negative controls; and gold nanoparticles functionalized with coumarin-PEG-thiol as the test preparation in serum free medium (SFM) and incubated for 24 hours. An additional control wells received only SFM. After the incubation time, the cells were washed once with sterile phosphate buffered saline (PBS, pH 7.4), followed by addition of 180 µl of DMEM per well. The plates were incubated for 4 hours after the addition of 20 µl of MTS solution to each well and the absorbance of the formed formazan product was read at 490 nm using a microplate reader. The absorbance values were converted to percentage viability against control and reported as mean and standard deviation.

*Fluorescence Confocal Microscopy Studies:* A known number of cells were grown on circular glass cover slips in a six-well plate containing DMEM. Once they had reached suitable confluency, the medium was replaced with SFM and the controls and test preparations were added. The final concentration of coumarin-PEG-thiol functionalized gold nanoparticles was 100 µg/ml per well and the samples were incubated for one hour. The cells were washed with sterile PBS twice and fixed with paraformaldehyde solution [0.5% (w/v) in PBS]. The cover slips were mounted (with cell side down) onto clean glass slides using Fluoromount G mounting medium. The fluorescent images were registered using an in-house built Keck 3-D Fusion microscope system using a 100x oil immersion objective.

## Results and Discussion

Hetero-bifunctional PEG having a thiol group on one terminus and conjugated with coumarin carbamate on the other was efficiently synthesized and purified. Gold nanoparticles were prepared by reduction of gold hypochlorite ($HAuCl_4$) with freshly-prepared trisodium citrate. As the gold nanoparticles are formed, the color of the dispersion changes from pale yellow to bright red. As can be seen from the Coulter particle size measurements and the TEM image in Figure 1, we can reproducibly make gold nanoparticles with a mean diameter of 10 nm that have a narrow size distribution and are spherical in shape.

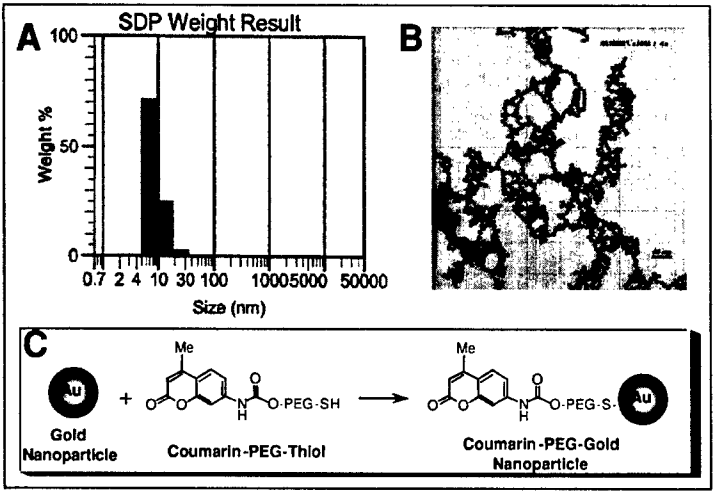


**Figure 1.** Coulter® particle size distribution (A), the transmission electron microscopy image of gold nanoparticles (B), and schematic illustration of the functionalization of gold nanoparticles with coumarin-poly(ethylene glycol)-thiol (C).

Cellular cytotoxicy studies in Figure 2 show that neither gold nanoparticles, gold nanoparticles finctionalized with methoxy-PEG-thiol (i.e., gold-PEG-OMe nanoparticles) nor coumarin-PEG-thiol functionalized gold nanoparticles were cytotoxic at the concentrations studied. In contrast, polyethyleneimine, a known cytotoxic compound, did reduce cell viability to 40% of the control at the same concentrations.

Although gold nanoparticles have been studied as very high efficient fluorescence quenchers [12], we observed that attachment of coumarin with the PEG spacer did significantly improve the fluorescence emission signal. This could be due to the fact that the dye was no longer in direct contact with the gold surface. Cellular uptake and distribution analysis using fluorescence confocal microscopy, as shown in Figure 3, show that the functionalized nanoparticles were efficiently internalized by non-specific endocytosis in mammalian tumor cells within one hour of incubation. The nanoparticles were found to have localized mainly in the cytosol and peri-nuclear region of the cells.

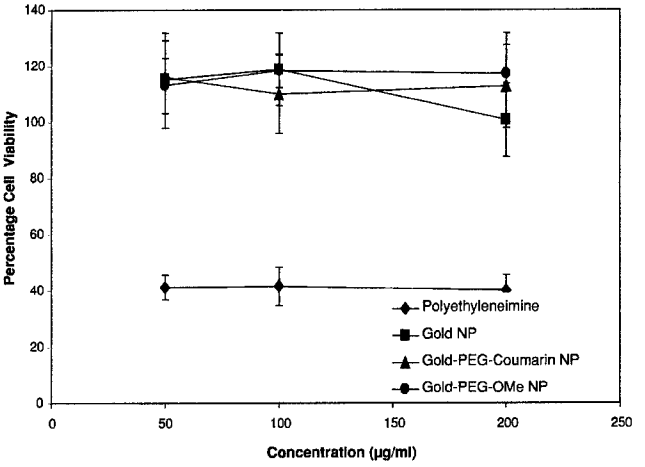


**Figure 2.** Cytotoxicity analysis of the controls and coumarin-poly(ethylene glycol)-thiol functionalized gold nanoparticles in MDA-MB-231 breast cancer cells. Bare gold nanoparticles and nanoparticles functionalized with methoxy-poly(ethylene glycol)-thiol were used as negative controls and polyethyleneimine (MW 10,000) was used as a positive control.

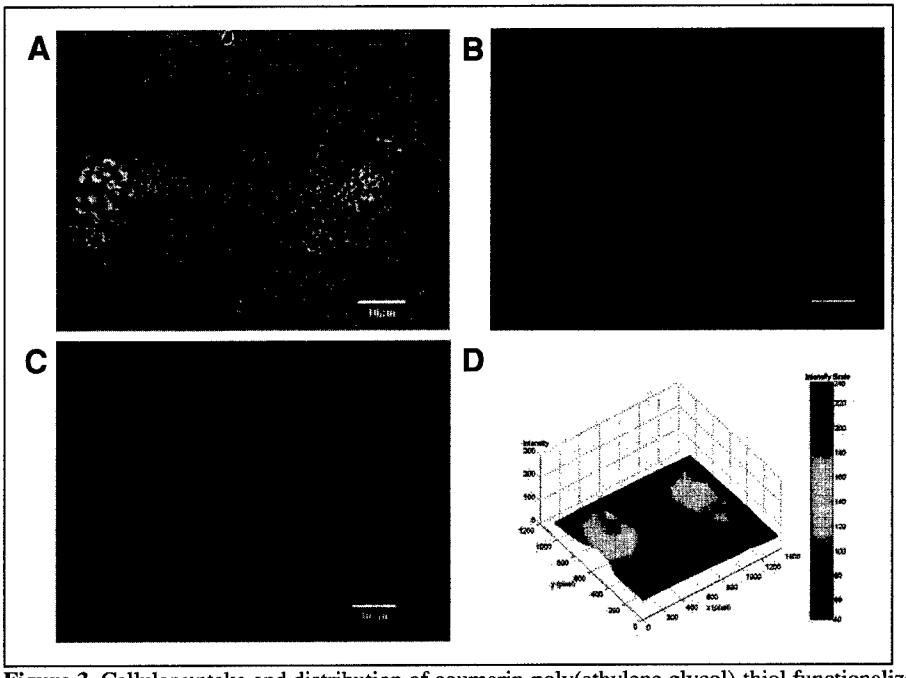


**Figure 3.** Cellular uptake and distribution of coumarin-poly(ethylene glycol)-thiol functionalized gold nanoparticles in MDA-MB-231 breast cancer cells. Differential interference contrast (A), epifluorescence (B), merged images (C), and 3-D plot of fluorescence intensity distribution as a functional of the X and Y coordinates was plotted (D). Original magnification was 100X.

## Conclusions

Coumarin-PEG-thiol was synthesized starting from PEG (MW 1,500) for attachment to gold nanoparticles through a hydrophilic spacer. The synthesis method produced the desired derivative at high efficiency. Gold nanoparticles, functionalized with coumarin through the PEG spacer were significantly fluorescent. Cytotoxicity measurements showed that these nanoparticles were essentially nontoxic to MDA-MB-231 breast cancer cells and could be efficiently internalized in the cells and were localized in the cytosol and peri-nuclear region within one hour of incubation.


## Acknowledgements

This study was supported by a grant RO1-CA095522 from the National Institutes of Health and by the Electronic Materials Research Institute of Northeastern University.


## References


1. Zandonella, C. *Nature* **423:** 10-12 (2003).
2. West, J.L. and N.J. Halas. *Curr. Opinion Biotech.*, **11:** 215-217 (2000).
3. Sahoo, S.K. and V. Labhasetwar. *Drug Disc. Today.*, **8:** 1112-1120 (2003).
4. LaVan, D.A., D.M. Lynn, and R. Langer. *Nature Revs.*, **1:** 77-84 (2002).
5. Goodman, C.M., C.D. McCusker, T. Yilmaz, and V.M. Rotello, *Bioconj. Chem.*, **15**: 897-900 (2004)
6. Hirsch, L.R., R.J. Stafford, J.A. Bankson, S.R. Shersen, B. Rivera, R.E. Price, J.D. Hazle, N.J. Halas, and J.L. West. *Proc. Natl. Acad. Sci., USA* **100:** 13549-13553 (2003).
7. Hainfeld, J.F., D.N. Slatkin, and H.M. Smilowitz, *Phys. Med. Biol.*, **49**: N309-N315 (2004)
8. Thomas, M and A. Klibanov. *Proc. Natl. Acad. Sci., USA* **100:** 9138-9143 (2003).
9. J-M Nam, C.S. Thaxton, and C.A. Mirkin, *Science*, **301:** 1884 (2003).
10. Potineni, A., D.M., Lynn, R. Langer, and M.M. Amiji. *J. Controlled Rel.*, **86:** 223-234 (2003).
11. Bouzide, A., and G. Sauve. *Org. Lett.*, **4:** 2329-2332 (2002).
12. Fan, C., S. Wang, J.W. Hong, G.C. Bazan, K.W. Plaxco, and A.J. Heeger. *Proc. Natl. Acad.*